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Genetic polymorphisms of the CYP1A1, GSTM1, and GSTT1 enzymes and their influence on cardiovascular risk and lipid profile in people who live near a natural gas plant

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The aim of this cross-sectional study was to see whether genetic polymorphisms of the enzymes CYP1A1, GSTM1, and GSTT1 are associated with higher risk of coronary artery disease (CAD) and whether they affect lipid profile in 252 subjects living near a natural gas plant, who are likely to be exposed to polycyclic aromatic hydrocarbons (PAHs). Fasting serum concentrations of biochemical parameters were determined with standard methods. Genetic polymorphisms of *CYP 1A1* rs4646903, rs1048943, rs4986883, and rs1799814 were genotyped with polymerase chain reaction-restriction fragment length polymorphism (PCR-RFPL), while *GSTM1* and *GSTT1* deletions were detected with multiplex PCR. Cardiovascular risk was assessed with Framingham risk score, and the subjects divided in two groups: >10% risk and ≤10% risk. The two groups did not differ in the genotype frequencies. MANCOVA analysis, which included lipid parameters, glucose, and BMI with sex, age, hypertension and smoking status as covariates, showed a significant difference between the *GSTT1*0* and *GSTT1*1* allele carriers (p=0.001). UNIANCOVA with same covariates showed that total cholesterol and triglyceride levels were significantly higher in *GSTT1*1* allele carriers than in *GSTT1*0* carriers (p<0.001 and p=0.006, respectively). Our findings suggest that CYP1A1, GSTM1, and GSTT1 polymorphisms are not associated with the higher risk of CAD, but that *GSTT1* affects lipid profile.

KEY WORDS: *cholesterol; cytochrome P-450; glutathione S transferase M1; glutathione S transferase T1; polymerase chain reaction; restriction fragment length polymorphism; triglycerides*

Coronary artery disease (CAD) is one of the most frequent causes of death in the world, yet we still do not know all the factors that increase CAD risk, and research has recently focused on different genetic, lifestyle, and environmental factors (1, 2).

Persistent organic compounds like polycyclic aromatic hydrocarbons (PAHs) can affect the expression of cytochrome *P450 (CYP*) and glutathione S-transferases (*GST*) genes (3). PAHs are environmental pollutants produced by fossil fuel combustion, industrial and natural emission, and many other sources (4). Humans are mostly exposed to PAHs through the use of materials that contain them, through exhaust inhalation, and through tobacco smoke (5).

Literature points to the involvement of PAHs in carcinogenesis as well as atherosclerosis through inflammatory process and DNA-adduct formation (6, 7). Furthermore, PAHs can affect the expression of cytochrome P450 (*CYP*) and glutathione S-transferase (*GST*) genes, which produce the respective first and second-phase biotransformational enzymes (8, 9).

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P4501A1 (CYP1A1) is important for the liver oxidation of lyophilic substrates like PAH (10) and have several polymorphisms: *m1*=3801 T>C (11); *m2*=2455 A>G (12); *m3*=3205 T>C (13) and *m4*=2453 C>A (14).

Through conjugation with glutathione GST participates in organism defence against mutations and oxidative stress that lead to atherosclerosis. As its activity depends on the *GST* gene polymorphisms, these may increase the risk of carcinogenesis and inflammatory processes (15).

There are three families of glutathione-S transferases: microsomal, mitochondrial, and cytosolic (15). The most frequent isoforms of the cytosolic family are alpha (GSTA), mu (GSTM), pi (GSTP), sigma (GSTS), omega (GSTO), zeta (GSTZ), and theta (GSTT). This is the family of enzymes that detoxify reactive electrophilic compounds but is also involved in lipid metabolism and biosynthesis of lipid-derived biogenic compounds (15). Human *GSTM1* and *GSTT1* genes exhibit a very common polymorphism that partially or completely deletes those genes and their enzymes (16).

Even though average PAH air pollution at the Molve (Croatia) natural gas processing plant does not seem to exceed regulatory limits, seasonal variations may trigger processes associated with gene expression and the risk of CAD. This may especially be true for *GST* polymorphisms

that increase organism sensitivity to genotoxic compounds. The (primary) aim of this study was to see whether genetic polymorphisms of the enzymes CYP1A1, GSTM1, and GSTT1 are associated with the higher risk of CAD and whether they affect lipid profile in people who may be exposed to polycyclic aromatic hydrocarbons (PAHs).

PARTICIPANTS AND METHODS

Study participants and design

The data and samples for this cross-sectional study were collected between 2007 and 2009. It included 252 adult participants from the general population who had been living near a natural gas plant in the rural community of Molve for over 10 years. The participants were recruited in collaboration with the local primary healthcare clinic and all who agreed to participate signed an informed consent form. The study was approved by the Ethics Committee of the Zagreb University School of Medicine.

Fasting blood samples were collected in collaboration with family medicine physicians. Whole blood was collected into the K_2EDTA tubes and serum separator Vacutainer tubes (Terumo Europe, Leuven, Belgium) for DNA extraction and serum separation. Sera were stored at -20 °C until analyses.

Family history of cardiovascular diseases and individual risk factors for atherosclerosis (hypertension, smoking, and alcohol consumption) was taken with a questionnaire. Smokers were defined as either ex-smokers or active smokers, while non-smokers were those who had never smoked. Height and weight were taken to calculate the body mass index (BMI). Blood pressure was measured in the sitting position, after 10-20 min of rest. Diabetes mellitus was determined according to the WHO diagnostic criteria (17) and history of insulin or oral glucose-lowering therapy.

Cardiovascular risk was assessed by calculating the Framingham risk score from the following information: age, sex, T-cholesterol, HDL-cholesterol, smoking status, systolic blood pressure, and blood pressure treatment (18). We used a web calculator available at *https://www.easycalculation.com/medical/framingham.php*. This tool is used to assess the risk of coronary heart diseases and/or severe outcomes like myocardial infarction or coronary death in the next 10 years. We determined three categories of cardiovascular risk for sever cardiovascular outcome in the next 10 years(19): above 20 %, between 10-20 % and below 10 %. As only a few participants ran the risk above 20 %, we merged them with the group with high risk of CAD (>10%). The second group ran low risk (≤10%). As the Framingham score algorithm tolerates the risk of up to 30 %, five participants were excluded from the analysis.

Blood serum analysis

Concentrations of total cholesterol, HDL-cholesterol, triacylglycerol, and glucose were determined on a COBAS INTEGRA 400+ analyser (Roche Diagnostics, Mannheim, Germany) following the standard procedure. LDL-cholesterol was calculated with the Friedwald equation.

DNA analysis

DNA was extracted with the salting-out method (20) and four *CYP1A1* genetic polymorphisms determined with polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP): m1=3801 T/C (rs4646903), m2=2455 A/G Ile/Val (rs1048943); m3=3205 T/C (rs4986883) and m4=2453 C/A Thr/Asp (rs1799814). PCR was performed using oligonucleotides from TibMolBiol (Berlin, Germany) created according the sequence from literature (21, 22).

PCR reactions were performed on an Eppendorf Mastercycler® 3350 (Eppendorf, Hamburg, Germany). The PCR-reaction mixture of 25 μL consisted of 500 ng of genomic DNA, 0.6 μmol L^{-1} of each primer, 10^{-3} μmol L^{-1} of $MgCl_2$, 0.25 μmol L^{-1} of each dNTP, and 0.75 U of FastStart TaqDNA Polymerase (Roche, Manheim, Germany). The reactions started with denaturation at 94 °C for 2 min. Each of the 35 cycles that followed consisted of 30 s at 94 °C, 30 s at 63 °C, and 1 min at 72 °C, followed by a 7-min extension at 72 °C. The 899-bp-long fragment (m1 & m3) was digested with *Msp* I (Roche Diagnostics, Manhem, Germany) to yield 693- and 206-bp-long fragments in the presence of the *m1* polymorphism and 802- and 97-bp-long fragments in the presence of the *m3* polymorphism. The 204-bp-long fragment (m2 & m4) was digested with *BsrDI* and *BsaI* (Fermentas International Inc., Burlington, Canada) to yield 149- and 55-bp-long fragments in the presence of the *m2* polymorphism and 139- and 65-bp-long fragments in the presence of the *m4* polymorphism. The fragments were detected on 2 % agarose gel stained with ethidium-bromide.

GSTM1 and *GSTT1* deletions (*GSTM1*0* and *GSTT1*0* alleles) were detected with the multiplex-PCR method with three pairs of nucleotides (TibMolBiol, Berlin, Germany) created according to the sequence from literature (16). In short, two pairs of oligonucleotides served to anneal the *GSTM1* and *GSTT1* fragments without deletion, while the third pair served to anneal the *GSTM4* fragment as control without deletion. The fragments were detected on 2 % agarose gel stained with ethidium-bromide. The 230- and 480-bp-long fragments corresponded to the respective *GSTM1* and *GSTT1* positive genotypes with at least one functional allele (*GSTM1*1* and *GSTT1*1*). The 157-bp-long fragments corresponded to *GSTM4* as an internal control. PCR reactions started with denaturation at 94 °C for 5 min, followed by 35 cycles, each consisting of 1 min at 94 °C, 1 min at 52 °C and 1 min at 72 °C, followed by a 7-min extension at 72 °C.

The PCR for every 10th sample was repeated and DNA concentrations adjusted. For all samples with unsuccessful PCR or RFLP we repeated the procedure.

Statistical analysis

For statistical analysis we used Statistica 12 (Tulsa, OK, USA). Categorical variables were tested with Pearson's chi-square test. Continuous variables were expressed as means with standard deviations or medians and 25th to 75th quartile, depending on distribution normality. For multiple testing with sex, age, diabetes mellitus, hypertension, and smoking status as covariates we used multivariate analysis of variance (MANCOVA). For this purpose triglycerides were log-transformed.

The level of significance was set at 0.05. To account for multiple ANOVAs we used Bonferroni correction due to multiple comparisons. The Bonferroni correction sets the significance cut-off at *α/11* for MANCOVA and *α/5* for UNIANCOVA.

RESULTS

Table 1 shows the biochemical and anthropometric information about the study participants by risk groups. As expected, they significantly differed in all risk factors. Besides, the high-risk group had significantly more diabetics, higher BMI, glucose, and triglyceride concentrations than the low-risk group.

However, the two groups did not differ in genotype frequencies of *CYP1A1m1, CYP1A1m2, CYP1A1m4, GSTM1,* and *GSTT1* (Table 1). According to Hardy-Weinberg proportions, genotypes are expected to be in equilibrium. The chi-square test *p* values for calculating the differences between the observed and expected genotype frequencies in the low-risk group were 0.459, 0.211, and 0.406 for *CYP1A1-m1, m2, and m4*, respectively. In the high-risk group, the *p* values for the same genotypes were 0.098, 0.952, and 0.789, respectively. As the frequency of rare homozygous genotypes for all three polymorphisms was low, we merged them with the frequencies of heterozygotes into one category as "rare allele carriers". *CYP1A1-m3* was not detected in any of the participants. There was no difference in frequencies of wild and rare allele carriers.

Table 2 shows the associations between biochemical and anthropometric parameters and the polymorphisms, except for the 18 participants with diabetes, because diabetes affects lipid profile. A significant difference in lipid levels was found with MANCOVA between the *GSTT1-0* and *GSTT1-1* allele carriers (p=0.001; F=3.65), while the *CYP1A1m1, CYP1A1m2, CYP1A1m4,* and *GSTM1* genotypes were not associated with lipid levels. Univariate

Table 1 *General biochemical and anthropometric characteristics and genotype frequencies in low- and high-risk groups (according to the Framingham risk score)*

Parameter*	Low-risk group (CAD risk Ł	High-risk group (CVD risk >10%) N=71	P
Age	43 (min-max:18-78)	63 (min-max:34-80)	<0.001
Sex: male, N (%)	39 (22.2)	47 (66.2)	<0.001
Hypertension: yes, N (%)	62 (35.2)	55 (77.5)	<0.001
Diabetes: yes, N (%)	5 (2.8)	13 (18.3)	<0.001
Smoking: yes, N (%)	60 (34)	37 (52)	0.001
*CYP1A1/m1 3801TT**, N	142	60	0.275
*CYP1A1/m1 3801TC+CC***, N	27 (25+2)	7 (6+1)	
*CYP1A1/m2 2455AA**, N	161	70	0.074
*CYP1A1/m2 2455AG+GG***, N	14 (13+1)	1 (1+0)	
*CYP1A1/m4 2453CC**, N	138	51	0.237
*CYP1A1/m4 2453CA+AA***+; N	37 (36+1)	20 (18+2)	
*GSTM1*1*, N	105	42	0.863
*GSTM1*0*, N	69	29	
*GSTT1*1*, N	123	51	0.395
*GSTT1*0*, N	54	17	
BMI, kg m^{-2}	27.32±5.23	30.15±5.27	<0.001
Total-cholesterol, mmol L^{-1}	5.41±1.03	5.88±1.17	0.002
HDL-cholesterol, mmol L^{-1}	1.30±0.37	1.04±0.32	<0.001
LDL-cholesterol, mmol L^{-1}	3.40±0.92	4.05±3.17	0.014
Triglycerides, mmol L^{-1}, median (25th; 75th quartile)	1.36 (0,96; 1.97)	1.93 (1.46; 3,52)	<0.001
Glucose, mmol L^{-1}, median (25th; 75th quartile)	4.90 (4.50; 5.50)	5.50 (4.80; 6,70)	<0.001

**wild allele carriers*
***rare-allele carriers*

analysis with the same covariates singled out total cholesterol and triglyceride concentrations as significantly higher in the positive *GSTT1* allele carriers than in the *GSTT1-0* carriers ($p<0.001$ and P=0.006, respectively). Triglyceride concentrations were also different between the *CYP1A1-m1* carriers and non-carriers, but the difference was marginally significant due to the Bonferroni correction.

Table 3 shows biochemical parameters and BMI in *CYP1A-m1* and *GSTT-1* polymorphism carriers who showed significant differences with the MANCOVA and UNIANCOVA analyses.

The *GSTT1*1* positive allele was significantly more common in subjects with serum triglyceride and total cholesterol concentrations above the 75th quartile than in subjects with these values below the 25th quartile. No such difference was observed for the *CYP1A1-m1* carrier frequencies (Table 4).

DISCUSSION

Our findings suggest that *CYP1A1, GSTT1*, or *GSTM1* genetic polymorphisms have not affected CAD risk in people who live near the natural gas processing plant. However, we have found a significant influence of the *GSTT1* deletion polymorphism on lipid parameters, serum triglyceride and total cholesterol in particular, after adjustment for sex, age, hypertension, and smoking status.

Our Molve population (Croatian northwest) has similar distribution of the *CYP 1A1-m1, GSTM1,* and *GSTT1* polymorphisms to the frequencies reported in other Croatian studies: the *GSTM1* and *GSTT1* deletion polymorphisms in pulmonary patients from Zagreb (23) and the *CYP1A1-m1 (3801 T/C) poly*morphism in urologic male patients form Osijek (24).

To the best of our knowledge these polymorphisms have not been investigated in relation to CAD in any Croatian population study. There are, however, several studies that investigated the association of the *CYP1A1-m1, m-2, m4, GSTM1*, and *GSTT1* polymorphisms with CAD in other countries. In Slovenian patients with diabetes mellitus the *GSTT1-0* deleted genotype was associated with an increased risk of carotid atherosclerosis. *GSTM1* and *GSTT1* polymorphisms, however, were not significantly associated with lipid parameters (25). In Italy, Manfredi et al. (26) found no significant differences in *CYP1A1* genotypes frequencies between smokers with CAD and without CAD, but did establish a significant association between combined *GSTM1* (null) plus *GSTT1*(null) genotypes and CAD risk. In Turkey, a significant CAD risk was established in the carriers of the *CYP1A1* variant plus *GSTT1* null genotype (27).

There are but a few studies that examine the association between lipids and *CYP1A1* polymorphisms, but none in European populations. In a Mexican study, *CYP1A1-m2* allele was significantly associated with high triglycerides

Table 2 *Effects of genetic polymorphisms on lipids, glucose, and BMI (diabetics excluded)*

	CYP1A1m1			CYP1A1m2			CYP1A1m4			GSTM1			GSTT1		
	F	df	P	F	df	P	F	df	P	F	df	P	F*	df	P
MANCOVA	1.72	6	0.313	1.32	6	0.250	1.14	6	0.336	1.30	6	0.257	3.65	6	0.001
UNIANCOVA															
Cholesterol	0.02	1	0.899	0.27	1	0.604	0.02	1	0.881	1.78	1	0.183	13.0	1	<0.001
HDL-cholesterol	<0.01	1	0.989	0.28	1	0.597	0.21	1	0.647	0.05	1	0.817	0.78	1	0.379
LDL-cholesterol	0.31	1	0.579	0.04	1	0.842	2.93	1	0.088	1.59	1	0.209	2.52	1	0.114
Triglycerides	5.71	1	0.017	2.48	1	0.117	0.36	1	0.546	3.03	1	0.084	7.61	1	0.006
Glucose	0.15	1	0.703	1.68	1	0.196	0.67	1	0.415	0.28	1	0.590	0.06	1	0.805
BMI	0.50	1	0.481	<0.01	1	0.958	1.72	1	0.190	144	1	0.231	0.13	1	0.721

MANCOVA - multivariate analysis of variance; UNIANCOVA - univariate analysis of variance

Table 3 *Biochemical parameters and BMI by CYP1A1 and GSTT-1 polymorphisms (diabetics excluded)*

	CYP1A1-1 3801 TT N=190	CYP1A1-1 3801 TC+CC N=30	GSTT1 *0-del. N=63	GSTT1 *1-func. N=164
	Mean±SD	Mean±SD	Mean±SD	Mean±SD
BMI, kg m^{-2}	28.1±5.6	27.6±5.5	28.1±5.7	28.0±5.5
CHOL, mmol L^{-1}	5.56±1.04	5.34±1.24	5.20±0.21	5.68±0.27
HDL-C, mmol L^{-1}	1.26±0.38	1.27±0.36	1.21±0.34	1.26±0.39
LDL-C, mmol L^{-1}	3.69±2.07	3.17±1.05	3.32±0.91	3.73±2.21
Glc, mmo L^{-1}	5.20±1.26	4.97±0.91	5.22±1.83	5.17±0.88
	Median (IQR)	Median (IQR)	Median (IQR)	Median (IQR)
TG, mmol/L	1.42 (0.99;2.02)	1.87 (1.15; 3.10)	1.27 (0.88; 1.78)	1.58 (1.01; 2.23)

Table 4 *Comparison between GSTT1 and CYP1A1 genotypes (with frequencies in the 1st and 4th quartile) for serum triglyceride and total cholesterol concentrations (diabetics excluded)*

Quartile	GSTT1*0-del. N (ratio)	GSTT1*1-func. N (ratio)	P (X^2- test)	Odds ratio (95% CI)
1st (TG≤0.99 mmol/L)	18 (0.38)	30 (0.62)	0.019	1
4th (TG≥2.11 mmol/L)	10 (0.17)	48 (0.83)		2.88 (1.17-7.07) P=0.021
1st (T-chol≤4.8 mmol/L)	23 (0.39)	36 (0.61)	0.002	1
4th (T-chol≥6.2 mmol/L)	9 (0.15)	53 (0.85)		3.76 (1.56-9.06) P=0.003
	CYP1A1-3801 TT	*CYP1A1-1 3801 TC+CC*		
1st (TG≤0.0.99 mmol/L)	41 (0.87)	6 (0.13)	0.231	1
4th (TG≥2.11 mmol/L)	43 (0.78)	12 (0.22)		1.91 (0.65-5.56) P=0.237

CI-confidence interval

(28), and in a Brazilian study in postmenopausal women the same allele was associated with high HDL-cholesterol (29). Unlike these studies, we have found no association between any of the *CYP1A1* polymorphisms and lipid parameters and neither has a Japanese study in women with osteopenia or osteoporosis (30).

Furthermore, unlike other studies in non-European populations, GSTT1 positive allele in our study was associated with hypertriglyceridemia (31-33).

As our results related to association of lipids and *GSTT1* polymorphism were opposite to literature data, we try to explain that with insights that are also presented in the literature. *Inter alia*, triacylglycerol metabolism includes activities of lipoprotein lipase, PPARγ – receptor and prostaglandin derivatives. As, active enzyme glutathione transferase catalyses the conjugation of prostaglandin derivative 15d-PG-J2 with glutathione, it therefore prevents PPARγ activation (34). Namely, 15d-PG-J2 may act as main endogenous ligand for the gamma isoform of the PPAR-receptors and if it is conjugate it cannot bind PPARγ receptor (34). Due the absence of PPARγ activation, one of the main it's roles, cannot be expressed. Active PPARγ pronounces triglyceride-lowering effects because it's activation induces adipose tissue LPL gene expression, resulting in a more efficient lipolysis and clearance of triglycerides from circulation (35, 36). Therefore we supposed that different conditions which leads to different genetic expression might be involved in lowering of lipid concentrations in GSTT1-0, null allele carriers.

The added value of this study is that it brings original population polymorphism data for the Molve area so that we can see how it stands in relation to other Croatian population studies and some other European populations, but could not compare lipid associations with the studied polymorphisms, as the data only exist for non-European populations.

The study is limited by its small sample, narrow geographical area, and lack of data about PAH exposure (such as urinary PAH metabolites) because this population lives near a natural gas processing plant.

In conclusion, genetic polymorphisms of the enzymes *CYP1A1, GSTM1*, and *GSTT1* are not associated with the higher risk of coronary artery disease (CAD), and only *GSTT1* seems to affect the lipid profile of the Molve population.

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Genski polimorfizmi enzima CYP1A, GSTM1 i GSTT1 i njihov utjecaj na rizik od kardiovaskularne bolesti i lipidni profil u ispitanika koji su nastanjeni u blizini crpilišta prirodnog plina

Cilj ovog presječnog istraživanja bio je ispitati moguću povezanost između genskih polimorfizama enzima i većeg rizika od koronarne arterijske bolesti (KAB) i promjena koncentracija lipida u krvi. U istraživanje su bila uključena 252 ispitanika koja više od deset godina prebivaju u blizini crpilišta prirodnog plina, zbog čega bi mogli biti izloženi utjecaju policikličkih aromatskih ugljikovodika. Koncentracije biokemijskih analita određene su standardnim metodama u serumu izdvojenome iz krvi uzete natašte. Genski polimorfizmi *CYP 1A1* rs4646903, rs1048943, rs4986883 i rs1799814 utvrđeni su pomoću lančane reakcije polimerazom i metode ispitivanja polimorfizama duljine restrikcijskih ulomaka (PCR-RFLP). Delecije gena *GSTM1* i *GSTT1* utvrđene su pomoću višestruke lančane reakcije polimerazom. Rizik od kardiovaskularnih bolesti izračunan je pomoću Framinghamove procjene rizika, na temelju kojega su ispitanici podijeljeni u dvije skupne: rizik > 10 % i rizik ≤ 10 %. Ove dvije skupine nisu se razlikovale prema učestalosti ispitivanih genotipova. Analiza MANCOVA, koja je uključivala lipidne analite, glukozu i indeks tjelesne mase te četiri kovarijata (spol, dob, hipertenziju i pušenje), pokazala je statistički značajnu razliku između nositelja GSTT1*0 i GSTT1*1 alela ($p = 0,001$). UNIANCOVA analiza s istim kovarijatama pokazala je da su vrijednosti kolesterola i triacilglicerola značajno više u nositelja *GSTT1*1* alela nego u nositelja *GSTT1*0* ($p < 0,001$ i $p = 0,006$). Na temelju naših rezultata može se pretpostaviti da polimorfizmi u genima za CYP1A1, GSTM1 i GSTT1 nisu povezani s većim rizikom od kardiovaskularnih bolesti, ali da polimorfizam u genu za GSTT1 može utjecati na lipidni profil.

KLJUČNE RIJEČI: *kolesterol; citokrom P-450; glutation-S- transferaza M1; glutation-S- transferaza T1; lančana reakcija polimerazom; polimorfizam duljine restrikcijskih ulomaka; triacilglicerol*